NON-TOXIC, NON-TOXIGENIC, NON-PATHOGENIC *FUSARIUM* EXPRESSION SYSTEM

This application is a continuation-in-part of Serial No. 08/726,105, filed October 4, 1996, which is a continuation-in-part of of Serial No. 08/404,678 filed March 15, 1995, which is a continuation-in-part of Serial No. 08/269,449 filed June 30, 1994, which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to host cells useful in the production of recombinant proteins. In particular, the invention relates to non-toxic, non-toxigenic, and non-pathogenic fungal host cells of *Fusarium* which can be used in the high-level expression of recombinant proteins, especially enzymes. The invention further relates to promoter and terminator sequences which may be used in such a system.

Description of the Related Art

The use of recombinant host cells in the expression of heterologous proteins has in recent years greatly simplified the production of large quantities of commercially valuable proteins, which otherwise are obtainable only by purification from their native sources. Currently, there is a varied selection of expression systems from which to choose for the production of any given protein, including prokaryotic and eukaryotic hosts. The selection of an appropriate expression system will often depend not only on the ability of the host cell to produce adequate yields of the protein in an active state, but also to a large extent may be governed by the intended end use of the protein.

Although mammalian and yeast cells have been the most commonly used eukaryotic hosts, filamentous fungi have now begun to be recognized as very useful as host cells for recombinant protein production. Examples of filamentous fungi which are currently used or

proposed for use in such processes are *Neurospora crassa, Acremonium chrysogenum, Tolypocladium geodes, Mucor circinelloides* and *Trichoderma reesei, Aspergillus nidulans, Aspergillus niger* and *Aspergillus oryzae*.

Certain species of the genus *Fusarium* have been used as model systems for the studies of plant pathogenicity and gene regulation such as *Fusarium oxysporum* (Diolez et al., 1993, Gene 131:61-67; Langin et al., 1990, Curr. Genet. 17:313-319; Malardier et al., 1989, Gene 78:147-156 and Kistler and Benny, 1988, Curr. Genet. 13:145-149), *Fusarium solani* (Crowhurst et al., 1992, Curr. Genet. 21:463-469), and *Fusarium culmorum* (Curragh et al., 1992, Mycol. Res. 97:313-317). These *Fusarium* sp. would not be suitable commercially for the production of heterologous proteins because of their undesirable characteristics such as being plant pathogens or because they produce unsafe levels of mycotoxin. Dickman and Leslie (1992, Mol. Gen. Genet. 235:458-462) discloses the transformation of *Gibberella zeae* with a plasmid containing *nit-2* of *Neurospora crassa*. The strain of *Gibberella zeae* disclosed in Dickman and Leslie is a plant pathogen and produces zearalenone, an estrogenic mycotoxin. Sanchez-Fernandez et al. (1991, Mol. Gen. Genet. 225:231-233) discloses the transformation of *Gibberella fujikoroi* carrying a *niaD* mutation with a plasmid containing the *Aspergillus niger niaD* gene.

An ideal expression system is one which is substantially free of protease and mycotoxin production, also substantially free of large amounts of other endogenously made secreted proteins, and which is capable of higher levels of expression than known host cells. The present invention now provides new *Fusarium* expression systems which fulfill these requirements.

SUMMARY OF THE INVENTION

The present invention relates to non-toxic, non-toxigenic, non-pathogenic recombinant *Fusarium* host cell in the section Discolor (also known as the section Fusarium) or a synonym or teleomorph thereof, comprising a nucleic acid sequence encoding a heterologous protein operably linked to a promoter. The host cells and methods of the present invention are unexpectedly more efficient in the recombinant production of certain fungal enzymes than are other known fungal species, such as *Aspergillus niger Aspergillus oryzae*, or *Fusarium oxysporum*.

The invention also relates to methods for production of heterologous proteins, comprising culturing a host cell of the present invention under conditions conducive to

expression of the protein, and recovering the protein from the culture. In a preferred embodiment, the protein is a fungal protein, most preferably a fungal enzyme. Using the method of the present invention, at least about 0.5 g heterologous protein/l host cell is produced.

The invention further relates to a promoter sequence derived from a gene encoding a *Fusarium oxysporum* trypsin-like protease or a fragment thereof having substantially the same promoter activity as said sequence. The sequence of the promoter is shown in SEQ ID NO:5.

Additionally, the invention relates to a terminator sequence derived from a gene encoding a *Fusarium oxysporum* trypsin-like protease or a fragment thereof having substantially the same terminator activity as said sequence. The sequence of the terminator is shown in SEQ ID NO:6.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an SDS-PAGE gel of secreted proteins in *Fusarium graminearum* (lane 1); *Aspergillus niger* (lane 2); and *Aspergillus oryzae* (lane 3). Lane 4 shows molecular weight markers.

Figure 2 shows the results of a protease assay on the following samples: *Aspergillus oryzae* (well 1); *Aspergillus niger* (well 2); *Fusarium graminearum* (well 3); empty well controls (wells 4-6).

Figure 3 shows the construction of plasmid pJRoy6.

Figure 4 shows SDS-PAGE analysis of the secretion of a trypsin-like protease (SP387) in a transformant of *F. graminearum* ATCC 20334. Lane 1: molecular size markers; lane 2: blank; lane 3: purified trypsin-like protease protein standard; lane 4: blank; lane 5: *F. graminearum* strain ATCC 20334 untransformed; lane6: blank; lane 7: *F. graminearum* strain ATCC 20334 transformed with plasmid pJRoy6; lane 8: blank; line 9: molecular size markers.

Figure 5 shows a restriction map of pJRoy20.

Figure 6 shows a restriction map of pDM151.

Figure 7 shows a restriction map of pDM155.

Figures 8A and 8B show the level of expression of CAREZYME® in *Fusarium graminearum* when DSM 151-4 is fermented in *Fusarium graminearum* from 20-160 hrs. Figure 8A shows the results of an assay for CAREZYME®. Figure 8B shows SDS-PAGE analysis of the production of CAREZYME® in said *Fusarium graminearum*. Lane 1:molecular

size markers; lane 2:20 hrs.; lane 3:50 hrs.; lane 4:70 hrs.; lane 5:90 hrs.; land 6:120 hrs.; lane 7:140 hrs.; lane 8:160 hrs.

Figures 9A and 9B show the level of expression of LIPOLASE® when DSM 155-10 is fermented in *Fusarium graminearum* from 20-160 hrs. Figure 9A shows the results of an assay for LIPOLASE®. Figure 9B shows SDS-PAGE analysis of the production of LIPOLASE® in said *Fusarium graminearum*. Lane 1: molecular size markers; lane 2: 20 hrs.; lane 3: 50 hrs.; lane 4: 60 hrs.; lane 5: 90 hrs.; lane 6: 120 hrs.; lane 7: 140 hrs.; lane 8: 160 hrs.

Figure 10 shows a restriction map of pCaHj418.

Figure 11 shows a restriction map of pDM148.

Figure 12 shows a restriction map of pDM149.

Figure 13 shows a restriction map of pMHan37.

Figure 14 shows a restriction map of pDM154.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to non-toxic, non-toxigenic, non-pathogenic recombinant *Fusarium* host cell in the section Discolor (also known as the section Fusarium) or a synonym or teleomorph thereof, comprising a nucleic acid sequence encoding a heterologous protein operably linked to a promoter.

The known species in the section Discolor include, but are not limited to, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sulphureum*, *Fusarium trichothecioides Fusarium venenatum*, most specifically, *Fusarium venatum Nirenberg sp. nov.*, and *Fusarium toruloseum*. Known teleomorphs of *Fusarium* of the section Discolor include, but are not limited to, *Gibberella gordonii*, *Gibberella cyanea*, *Gubberella pulicaris*, and *Gibberella zeae*.

Fusarium strains are characterized by mycelium extensive and cotton-like in culture, often with some tinge of pink, purple or yellow in the mycelium on solid medium. Conidiophores are variable slender and simple, or stout, short, branched irregularly or bearing a whorl of phialides, single or grouped into sporodochia. Conidia are principally of two kinds, often held in small moist heads: macroconidia several-celled, slightly curved or bent at the pointed ends, typically canoe-shaped and microconidia which are one celled, ovoid or oblong,

borne singly or in chains. Some conidia are intermediate, 2 or 3 celled, oblong or slightly curved.

As defined herein, "non-toxic" means that the host cell does not act as a poison to plants or animals. For example, a *Fusarium* host cell would be considered non-toxic if about 14 days after injecting about 5 mice with a dose of about 20 ml of (1:1 diluted) 3 day old *Fusarium* culture medium/kg body wt./mouse, none of the mice died as a result of *Fusarium* treatment. As defined herein, "non-toxigenic" means that the host cells are essentially free of mycotoxin as determined by standard analytical methods such as HPLC analysis. For example, an amount of *Fusarium* grown on 2 x 9 cm petri dishes containing solid nutrient medium may be extracted with organic solvents and 0.5% of the extract may be injected into an HPLC for analysis. The absence of known mycotoxins would be inferred by the absence of detectable HPLC peaks at positions known for mycotoxin standards. As defined herein, "non-pathogenic" means that the host cells do not cause significant disease in healthy plants or healthy animals. For example, a *Fusarium* sp. that is pathogenic to plants can show a fungal invasion of the xylem tissue of the plant and result in the disease state characterized by typical wilt symptoms. As defined herein, a "heterologous protein" is a protein which is not native to the host cell, or a native protein in which modifications have been made to alter the native sequence or a native protein whose expression is quantitatively altered as a result of a manipulation of a native regulatory sequence required for the expression of the native protein, such as a promoter, a ribosome binding site, etc. or other manipulation of the host cell by recombinant DNA techniques. The nucleic acid sequence is operably linked to a suitable promoter sequence, which is capable of directing transcription of the nucleic acid sequence in the chosen host cell.

In a specific embodiment, the host cells of the present invention are of the species *Fusarium graminearum* which is characterized by the following features. Conidia: Microconidia are absent. Macroconidia are distinctly septate, thick walled, straight to moderately sickle-shaped, unequally curved with the ventral surface almost straight and a smoothly arched dorsal surface. The basal cell is distinctly foot-shaped. The apical cell is cone-shaped or constricted as a snout. Conidiophores: unbranched and branched monophialides. Chlamydospores:are generally very slow to form in culture:when they do occur, they most often form in the macroconidia but may also form in the mycelium. Colony morphology: on PDA, growth is rapid with dense aerial mycelium that may almost fill the tube and is frequently yellow to tan with the margins white to carmine red. Red-brown to orange

sporodochia, if present, are sparse, often appearing only when the cultures are more than 30 days old. The undersurface is usually carmine red. This fungus produces the most cylindrical (dorsal and ventral surfaces parallel) macroconidia of any species of the section Discolor.

In a most specific embodiment, the *Fusarium* strain has been deposited with the American Type Culture Collection and assigned the number ATCC 20334 and has been identified as *Fusarium graminearum* Schwabe IMI 145425 in U.S. Patent No. 4,041,189. The *Fusarium* strain may also be derivatives and mutants which are similarly non-toxic, non-toxigenic, and non-pathogenic, e.g. those taught in U.S. Patent No. 4,041,189.

It will be understood that throughout the specification and claims the use of the term "*Fusarium graminearum*" refers not only to organisms encompassed in this species, but also includes those species which have previously been or currently are designated as other species in alternate classification schemes, but which possess the same morphological and cultural characteristics defined above, and may be synonymous to *F. graminearum*. These include but are not limited to *Fusarium roseum, F. roseum* var. *graminearum, Gibberella zeae*, or *Gibberella roseum, Gibberella roseum* f. sp. *cerealis*.

The skilled artisan will also recognize that the successful transformation of the host species described herein is not limited to the use of the vectors, promoters, and selection markers specifically exemplified. Generally speaking, those techniques which are useful in transformation of *F. oxysporum, F. solani* and *F. culmorum* are also useful with the host cells of the present invention. For example, although the *amdS* selection marker is preferred, other useful selection markers include the *argB* (*A. nidulans* or *A. niger*), *trpC* (*A. niger* or *A. nidulans*), *pyrG* (*A. niger, A. oryzae* or *A. nidulans*), *niaD* (*A. nidulans, A. niger,* or *F. oxysporum*), and *hygB* (*E. coli*) markers. The promoter may be any DNA sequence that shows strong transcriptional activity in these species, and may be derived from genes encoding both extracellular and intracellular proteins, such as amylases, glucoamylases, proteases, lipases, cellulases and glycolytic enzymes. Examples of such promoters include but are not limited to *A. nidulans amdS* promoter or promoters from genes for glycolytic enzymes, e.g., TPI, ADH, GAPDH, and PGK. The promoter may also be a homologous promoter, i.e., the promoter for a gene native to the host strain being used. The promoter sequence may also be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the promoter sequence with the gene of choice or with a selected signal peptide or preregion.

The promoter sequence may be derived from a gene encoding a *Fusarium oxysporum* trypsin-like protease or a fragment thereof having substantially the same promoter activity as

said sequence. The sequence of the promoter is shown in SEQ ID NO:5. The invention further encompasses nucleic acid sequences which hybridize to the promoter sequence shown in SEQ ID NO:5 under the following conditions:presoaking in 5X SSC and prehybridizing for 1 hr. at about 40°C in a solution of 20% formamide, 5X Denhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 ug denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100 uM ATP for 18 hrs. at about 40°C, followed by a wash in 0.4X SSC at a temperature of about 45°C, or which have at least about 90% homology and preferably about 95% homology to SEQ ID NO:5, but which have substantially the same promoter activity as said sequence. In another embodiment, the promoter may be a sequence comprising a large number of binding sites of AreA, a positive regulator of genes expressed during nitrogen limitation; these sites are referred to as *nit-2* in *Neurospora crassa* (Fu and Marzlus, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:5331-5335). The promoter sequence may be modified by the addition or substitution of such AreA sites.

Terminators and polyadenylation sequences may also be derived from the same sources as the promoters. In a specific embodiment, the terminator sequence may be derived from a gene encoding a *Fusarium oxysporum* trypsin-like protease or a fragment thereof having substantially the same terminator activity as said sequence. The sequence of the terminator is shown in SEQ ID NO:6. The invention further encompasses nucleic acid sequences which hybridize to the terminator sequence shown in SEQ ID NO:6 under the following conditions:presoaking in 5X SSC and prehybridizing for 1 hr. at about 40°C in a solution of 20% formamide, 5X Denhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 ug denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100 uM ATP for 18 hrs. at about 40°C, followed by a wash in 0.4X SSC at a temperature of about 45°C, or which have at least about 90% homology and preferably about 95% homology to SEQ ID NO:5, but which have substantially the same terminator activity as said sequence.

Enhancer sequences may also be inserted into the construct.

To avoid the necessity of disrupting the cell to obtain the expressed product, and to minimize the amount of possible degradation of the expressed product within the cell, it is preferred that the product be secreted outside the cell. To this end, in a preferred embodiment, the gene of interest is linked to a preregion such as a signal or leader peptide which can direct the expressed product into the cell's secretory pathway. The preregion may be derived from genes for any secreted protein from any organism, or may be the native preregion. Among

useful available sources for such a preregion are a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the alpha-factor from *Saccharomyces cerevisiae*, or the calf prochymosin gene. The preregion may be derived from the gene for *A. oryzae* TAKA amylase, *A. niger* neutral alpha-amylase, *A. niger* acid stable α-amylase, *B. licheniformis* α-amylase, the maltogenic amylase from *Bacillus* NCIB 11837, *B. stearothermophilus* α-amylase, or *B. licheniformis* subtilisin. An effective signal sequence is the *A. oryzae* TAKA amylase signal, the *Rhizomucor miehei* aspartic proteinase signal and the *Rhizomucor miehei* lipase signal. As an alternative, the preregion native to the gene being expressed may also be used, e.g., in SEQ ID NO:4 between amino acids -24 and -5.

The gene for the desired product functionally linked to promoter and terminator sequences may be incorporated in a vector containing the selection marker or may be placed on a separate vector or plasmid capable of being integrated into the genome of the host strain. Alternatively, the vectors used may be capable of replicating as linear or circular extrachromosomal elements in the host cell. These types of vectors include for example, plasmids and minichromosomes. The vector system may be a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be integrated into the genome. Vectors or plasmids may be linear or closed circular molecules.

The host cell may be transformed with the nucleic acid encoding the heterologous protein using procedures known in the art such as transformation and electroporation (see, for example, Fincham, 1989, Microbial Rev. 53:148-170).

The recombinant host cell of the present invention may be cultured using procedures known in the art. Briefly, the host cells are cultured on standard growth medium such as those containing a combination of inorganic salts, vitamins, a suitable organic carbon source such as glucose or starch, any of a variety of complex nutrients sources (yeast extract, hydrolyzed casein, soya bean meal, etc.). One example is FP-1 medium (5% soya bean meal, 5% glucose, 2% K_2HPO_4, 0.2% $CaCl_2$, 0.2% $MgSO_4 7H_2O$ and 0.1% pluronic acid (BASF)). The fermentation is carried out at a pH of about 4.5-8.0, and at a temperature of about 20-37°C for about 2-7 days.

The present host cell species can be used to express any prokaryotic or eukaryotic heterologous protein of interest, and is preferably used to express eukaryotic proteins. Of particular interest for these species is their use in expression of heterologous proteins, especially fungal enzymes. The novel expression systems can be used to express enzymes such

as catalase, laccase, phenoloxidase, oxidase, oxidoreductases, cellulase, xylanase, peroxidase, lipase, hydrolase, esterase, cutinase, protease and other proteolytic enzymes, aminopeptidase, carboxypeptidase, phytase, lyase, pectinase and other pectinolytic enzymes, amylase, glucoamylase, alpha-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, mannosidase, isomerase, invertase, transferase, ribonuclease, chitinase, mutanase and deoxyribonuclease.

In a specific embodiment, the enzyme is an alkaline protease, e.g., a *Fusarium oxysporum* pre-pro-trypsin gene. In a most specific embodiment, the genomic sequence is shown in SEQ ID NO:3 and the protein sequence is shown in SEQ ID NO:4.

In another specific embodiment, the enzyme is an alkaline endoglucanase, which is immunologically reactive with an antibody raised against a highly purified ~43 kD endoglucanase derived from *Humicola insolens*, DSM 1800, or which is a derivative of the ~43 kD endoglucanase exhibiting cellulase activity (cf. WO 91/17243). The endoglucanase, hereinafter referred to as "CAREZYME®" may be encoded by a gene shown in SEQ ID NO:7 and may have a protein sequence shown in SEQ ID NO:8. The enzyme may also be a CAREZYME® variant.

In yet another specific embodiment, the enzyme is a 1,3-specific lipase, hereinafter referred to as LIPOLASE®. The enzyme may be encoded by the DNA sequence shown in SEQ ID NO:9 and may have an amino acid sequence shown in SEQ ID NO:10. The enzyme may also be a LIPOLASE® variant, e.g., D96L, E210K, E210L (see WO 92/05249).

It will be understood by those skilled in the art that the term "fungal enzymes" includes not only native fungal enzymes, but also those fungal enzymes which have been modified by amino acid substitutions, deletions, additions, or other modifications which may be made to enhance activity, thermostability, pH tolerance and the like. The present host cell species can also be used to express heterologous proteins of pharmaceutical interest such as hormones, growth factors, receptors, and the like.

The present invention will be further illustrated by the following non-limiting examples.

EXAMPLES

Example 1 - *Fusarium graminearum* ATCC 20334 Secretes Only a Low Level of Protein

Conidial spore suspensions of *Fusarium graminearum* strain ATCC 20334, an *A. oryzae*, and *A. niger* are inoculated into 25 ml of YPD medium (1% yeast extract (Difco), 2% bactopeptone (Difco), 2% glucose) in a 125 ml shake flask and incubated at 30°C at 300 rpm

for 5 days. Supernatant broths from the cultures are harvested by centrifugation. A total of 10 μl of each sample are mixed with 10 μl 0.1 M dithiothreitol (Sigma) and 10 μl of loading buffer (40 mM Tris base, 6% sodium dodecyl sulfate, 2.5 mM EDTA, 15% glycerol, 2 mg/ml bromocresol purple). The samples are boiled for 5 minutes and run on a 4-12% polyacrylamide gel (Novex). The proteins are visualized by staining with Coomassie Blue. The results (Figure 1) show that *Fusarium graminearum* strain ATCC 20334 produces very little secreted protein.

Example 2 - *Fusarium graminearum* ATCC 20334 Secretes Only a Low Level of Proteases

A total of 40 μl of culture broths from *Fusarium graminearum* strain ATCC 20334, *A. oryzae*, and *A. niger* (see Example 1) are each pipetted into wells that are cut into a casein agar plate (2% non-fat dry milk (Lucerne), 50 mM Tris-HCl pH=7.5, 1% noble agar (Difco)). The plates are incubated at 37°C for 5 hours and the zones of protein hydrolysis are observed. The results (Figure 2) show that *Fusarium graminearum* strain ATCC 20334 broth contains very little proteolytic activity.

Example 3 - Cloning of *Fusarium oxysporum* Genomic Prepro-trypsin Gene

A genomic DNA library in lambda phage is prepared from the *F. oxysporum* genomic DNA using methods such as those described found in Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY. A total of 50 μg genomic DNA are digested in a volume of 200 μl containing 10 mM Tris (pH=7.5), 50 mM NaCl, 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol, and 4 units restriction enzyme Sau3A for one minute at 37°C. Partially digested DNA of molecular size 10-20 kb is isolated by agarose gel electrophoresis, followed by electroelution into dialysis membrane and concentration using an Elutip-D column (Schleicher and Schuell). One μg of lambda arms of phage of EMBL4 that had been cut with restriction enzyme *Bam*H1 and treated with phosphatase (Clonetech) is ligated with 300-400 μg Sau3A cut genomic DNA in a volume of 25 μl under standard conditions (see Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY). Lambda phage are prepared from this ligation mix using a commercially available kit (Gigapack Gold II, Stratagene) following the manufacturers directions.

The plating of *ca.* 15,000 recombinant lambda phage and the production of filter lifts (to Hybond N^+ filters, Amersham) are performed using standard methods (Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY). The filters are processed for hybridization with a Genius Kit for nonradioactive nucleic acids detection

(Boehringer Mannheim) using standard methods (Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY). The DNA used as a probe is a 0.75 kb digoxygenin (DIG) labeled PCR fragment of the entire coding region of the *F. oxysporum* trypsin-like protease (hereinafter referred to as SP387) gene present in plasmid pSX233, which has been deposited with the NRRL under the accession number of NRRL B-21241. The primers for the PCR reaction are 5'-tgcggatccATGGTCAAGTTCGCTTCCGTC (forward primer; SEQ ID NO:1) and 5'-gacctcgagTTAAGCATAGGTGTCAATGAA (reverse primer; SEQ ID NO:2). In both primers, the lower case characters represent linker sequences and the upper case characters correspond to the coding region of the SP387 gene. To perform the PCR, 25 ng of a 907 bp BamH1/Xba1 DNA fragment containing the SP387 gene from plasmid pSX233 are mixed with 68 pmoles of each forward and reverse primer.

The mixture of the DNA fragment and primers is made up to an 80 μl volume in 1X Taq Buffer/1X DIG labelling Mix/5 units Taq (Boehringer Mannheim). The reaction conditions are 95°C, 3 minutes, then 35 cycles of [95°C 30 seconds, 50°C 1 minute, 72°C 1 minute]. The DNA sequence derived by PCR from the *F. oxysporum* trypsin-like protease is shown in SEQ ID NO:3. The phage plaques are screened with the DIG labeled probe using a modification (Engler and Blum, 1993, Anal. Biochem. 210:235-244) of the Genius kit (Boehringer Mannheim). Positive clones are isolated and purified by a second round of plating and hybridization. Recombinant lambda phage containing the *F. oxysporum* trypsin-like protease gene are prepared and DNA is isolated from the phage using a Quiagen lambda midi preparation kit (Quiagen).

Example 4 - Construction of Expression Plasmid pJRoy6

Restriction mapping, Southern blotting, and hybridization techniques (Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, NY) are used to identify a 5.5 kb Pst1 restriction enzyme fragment from one of the recombinant phage that contains the *F. oxysporum* trypsin-like protease coding gene and flanking DNA sequences. This 5.5 kb Pst1 fragment is subcloned into Pst1 digested pUC118 and the plasmid is designated pJRoy4 (see Figure 3). Plasmid pJRoy4 is digested with restriction enzyme EcoR1 and a 3.5 kb EcoR1 fragment containing the SP387 gene and the 43 bp EcoR1/Pst1 region of the pUC118 polylinker is isolated and subcloned into the vector pToC90 to create plasmid pJRoy6 (Figure 3).

Example 5 - Construction of SP387 Expression Cassette

An expression cassette (pJRoy20) containing the SP387 promoter and terminator joined by a BamH1 site in pUC118 is constructed. An *E. coli* strain containing pJRoy20 has been deposited with the NRRL. The promoter fragment is generated by digesting the SP387 vector pJRoy6 with EcoR1 (which cuts at -1200) and with Nco1 (which cuts at the translational start site, see Figure 5). The terminator sequence (bp 2056-3107 in Figure 5) is generated by PCR amplification using the following oligonucleotides:

FORWARD

5'gcacaccatggtcgctggatccATACCTTGTTGGAAGCGTCG3' (SEQ ID NO:11)

REVERSE

5'atcggagcatgcggtaccgtttaaacgaattcAGGTAAACAAGATATAATTTTCTG 3' (SEQ ID NO:12)

Letters in large case are complementary to SP387 terminator DNA, while lower case letters are tails containing engineered restriction sites.

After digestion with Nco1 and Sph1, the resulting amplification product containing the terminator flanked by Nco1 and BamH1 sites on the 5' end, and flanked by EcoR1, Pme1, Kpn1 and Sph1 sites on the 3' end is isolated. A 3-way ligation between the promoter fragment, the terminator fragment and Kpn1/Sph1 cut pUC118 is performed to generate pJRoy20 (see Figure 5).

Example 6 - CAREZYME® Constructs

The EcoRV site at -15 in the SP387 promoter, and the Nco1 site present at +243 in the CAREZYME® coding region are utilized to create an exact fusion between the SP387 promoter and the CAREZYME® gene. A PCR fragment containing -18 to -1 of the SP387 promoter directly followed by -1 to + 294 of the CAREZYME® gene is generated from the CAREZYME® vector pCaHj418 (see Figure 10) using the following primers:

FORWARD

EcoRV

5'ctcttggatatctatctcttcaccATGCGTTCCTCCCCCCTCCT3' (SEQ ID NO:13)

REVERSE

5'CAATAGAGGTGGCAGCAAAA 3' (SEQ ID NO:14)

Lower case letters in the forward primer ar bp -24 to -1 of the SP387 promoter, while upper case letters are bp 1 to 20 of CAREZYME®.

The PCR conditions used are:95°C, 5 min. followed by 30 cycles of [95°C,30sec., 50°C, 1 min., 72°C, 1 min.]. The resulting 0.32 kb fragment is cloned into vector pCRII using Invitrogen's TA cloning kit resulting in pDM148 (see Figure 11). The 0.26 kb EcoRV/NcoI fragment is isolated from pDM148 and ligated to the 0.69 kb NcoI/BglII fragment from pCaHj418 and cloned into EcoRV/BamHI digested pJRoy20 to create pDM149 (see Figure 12). The 3.2 kb EcoRI CAREZYME® expression cassette (SP387 promoter/CAREZYME®/SP387 terminator) is isolated from pDM149 and cloned into the EcoRI site of pToC90 to create pDM151 (see Figure 6). Expression construct pDM151 contains both the expression cassette and the *amdS* selectable marker. An *E. coli* strain containing pDM151 has been deposited with the NRRL.

Example 7 - LIPOLASE® Constructs

The EcoRV site at -15 in the SP387 promoter, and the Sac1 site at +6 in the LIPOLASE® coding region are utilized to create an exact fusion between the SP387 promoter and the LIPOLASE® gene. An adapter containing the final 15 bp of the SP387 promoter followed by the first 6 bp of the LIPOLASE® coding region is constructed and is shown below.

EcoRV	SacI
atctatctcttcaccATGAGGAGCT	(SEQ ID NO:15)
tagatagagaagtggTACTCC	(SEQ ID NO:16)

A 0.9 kb SacI/BamHI fragment of the LIPOLASE® cDNA gene is isolated from the A.oryzae expression construct pMHan37 (see Figure 13). The EcoRV/SacI adapter and SacI/BamHI LIPOLASE® fragment are ligated and cloned into EcoRV/BamHI digested pJRoy20 to create plasmid pDM154 (see Figure 14). The 3.2 kb KpnI LIPOLASE® expression cassette (SP387 promoter/LIPOLASE®/SP387 terminator) is isolated from pDM154 and cloned into the KpnI site of pToC90 to create plasmid pDM155 (see Figure 7). Expression construct pDM155

contains both the LIPOLASE® expression cassette and the *amdS* selectable marker. An *E. coli* strain containing pDM151 has been deposited with the NRRL.

Example 8 - Transformation of *F. graminearum*

Fusarium graminearum strain ATCC 20334 cultures are grown on 100 x 15 mm petri plates of Vogels medium (Vogel, 1964, Am. Nature 98:435-446) plus 1.5% glucose and 1.5% agar for 3 weeks at 25°C. Conidia (approximately 10^8 per plate) are dislodged in 10 ml of sterile water using a transfer loop and purified by filtration through 4 layers of cheesecloth and finally through one layer of miracloth. Conidial suspensions are concentrated by centrifugation. Fifty ml of YPG (1% yeast extract (Difco) 2% bactopeptone (Difco), 2% glucose) are inoculated with 10^8 conidia, and incubated for 14 h at 20°C, 150 rpm. Resulting hyphae are trapped on a sterile 0.4 μm filter and washed successively with sterile distilled water and 1.0 M $MgSO_4$. The hyphae are resuspended in 10 ml of Novozym® 234 (Novo Nordisk) solution (2-10 mg/ml in 1.0 M $MgSO_4$) and digested for 15-30 min at 34°C with agitation at 80 rpm. Undigested hyphal material is removed from the resulting protoplast suspension by successive filtration through 4 layers of cheesecloth and through miracloth. Twenty ml of 1M sorbitol are passed through the cheesecloth and miracloth and combined with the protoplast solution. After mixing, protoplasts (approximately 5 x 10^8) are pelleted by centrifugation and washed successively by resuspension and centrifugation in 20 ml of 1M sorbitol and in 20 ml of STC (0.8 m sorbitol, 50 mM Tris-HCl pH=8.0, 50 mM $CaCl_2$). The washed protoplasts are resuspended in 4 parts STC and 1 part SPTC (0.8M sorbitol, 40% polyethylene glycol 4000 (BDH), 50 mM Tris-HCl pH=8.0, 50 mM $CaCl_2$) at a concentration of 1-2 x 10^8/ml. One hundred μl of protoplast suspension are added to 5 μg pJRoy6 and 5 μl heparin (5 mg/ml in STC) in polypropylene tubes (17 x 100 mm) and incubated on ice for 30 min. One ml of SPTC is mixed gently into the protoplast suspension and incubation is continued at room temperature for 20 min. Protoplasts are plated on a selective medium consisting of Cove salts (Cove, D.J., 1966, Biochem. Biophys. Acta 113:51-56) plus 10 mM acetamide, 15 mM $CsCl_2$, 2.5% noble agar (Difco) and 1.0 M sucrose using an overlay of the same medium with 0.6 M sucrose and 1.0% low melting agarose (Sigma). Plates are incubated at 25°C and transformants appeared in 6-21 days.

Example 9 - Expression of trypsin-like protease in *Fusarium graminearum*

Transformants are transferred to plates of COVE2 medium (same as COVE medium above without the cesium chloride and replacing the 1.0 M sucrose with a concentration of 30 g/l) and grown for 3 or more days at 25°C. Twenty five ml aliquots of FP-1 medium (5% soya bean meal, 5% glucose 2% K_2HPO_4, 0.2% $CaCl_2$, 0.2% $MgSO_4.7H_2O$ and 0.1% pluronic acid (BASF)) in 150 ml flasks are inoculated with approximately 1 cm agar plugs from COVE2 plate cultures and incubated for 6 days at 30°C with agitation (150 rpm). Supernatant broth samples are recovered after centrifugation and subjected to SDS-PAGE analysis as follows. Thirty μl of each broth is mixed with 10 μl SDS-PAGE sample buffer (1 ml 0.5 M Tris pH=6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 0.8 M dithiothreitol, 0.2 ml 1% bromophenol blue), 2 μl of 2% PMSF (Sigma) in isopropanol, and 2 μl glycerol. The samples are placed in a boiling water bath for 4 minutes and 40 μl of each are run on a 10-27% polyacrylamide gel (Novex). The gels are stained and destained with Coomassie dye using standard methods. The expression level of the trypsin-like protease has been determined to be $\geq$ 0.5 g/l.

Example 10 - Enzyme assays

A. CAREZYME®

Buffer: Sodium phosphate (50 mM, pH 7.0)

Substrate: AZCL-HE cellulose (Megazyme) at 2 mg/ml buffer

Enzyme std: 100 mg of CAREZYME® standard (10,070 ECU/g) is dissolved in 1 ml buffer and stored at -20°C. This stock is diluted 1:100 in buffer immediately prior to use in enzyme assays. The assay range is 0.5 - 5.0 ECU/ml. A conversion factor of 650,000 ECU/g CAREZYME® is used.

Substrate solution (990 μl) is added to sample wells of a 24-well microtiter plate. Ten μl of CAREZYME® sample (diluted in buffer to produce activity of between 0.5 and 10 ECU/ml.) are added to the substrate. Reactions are incubated for 30 minutes at 45°C with vigorous shaking, and next centrifuged at 4°C for 5 minutes at 5,000 rpm. Two hundred μl of supernatant are transferred to a 96-well microtiter plate and the absorbance at 650 nm is measured.

B. LIPOLASE® Assay

Buffer: 0.1M MOPS, pH 7.5 containing 4 mM $CaCl_2$

Substrate: 10 mL p-nitrophenyl butyrate (pNB)

in 1 ml DMSO;

Add 4 ml buffer to substrate in DMSO

*Stock concentration = 11.5 mM in 20% DMSO

Enzyme std: LIPOLASE® (23,100 LU/g) is dissolved at 1000 LU/ml in 50% glycerol and stored at -20°C. This stock is diluted 1:100 in buffer immediately prior to assay. The assay range is 0.125 to 3.0 LU/ml.

100 μl pNB stock solution is added to 100 μl of appropriately diluted enzyme sample. Activity (mOD/min) is measured at 405 nm for 5 min at 25°C.

C. SP387 Assay

L-BAPNA substrate is prepared by dilution of a 0.2 M stock solution of L-BAPNA (Sigma B3133) in dimethyl sulfoxide (stored frozen) to 0.004 M in buffer (0.01 M dimethylglutaric acid (Sigma), 0.2 M boric acid and 0.002 M calcium chloride, adjusted to pH 6.5 with NaOH) just prior to use. One μl of culture was centrifuged (145000 x g, 10 min). A 100 μl aliquot of diluted culture broth is added to 100 μl substrate in a 96 well microtiter plate. Absorption change at 405 nm is assayed at 30 second intervals for 5 min. at 25°C using an ELISA reader. Results are calculated relative to a purified SP387 standard.

Example 11 - Expression of CAREZYME®

Twenty-three transformants of pDM151 are purified, cultured in shake flasks on soy/glucose medium and assayed for CAREZYME® activity after 9 days (Table 1-see below). Four transformants express CAREZYME® at a level of approximately 50-100 mg/L. Transformant pDM151-4 is cultured in small scale fermentors using the conditions developed for SP387 production (see Example 9). Approximately 6.0 g/L of CAREZYME® is evident after 7 days (Figure 8A). CAREZYME® comprised greater than 90% of secreted proteins based on SDS gel electrophoresis (Figure 8B).

TABLE I

Transformant #	EMU/ml	mg/L
pDM 151.3 - 4	58.2	90
pDM 151.3 - 5	0	0

pDM 151.3 - 6	0	0
pDM 151.3 - 10	0	0
pDM 151.3 - 11	2.46	4
pDM 151.3 - 12	0	0
pDM 151.3 - 13	12.2	19
pDM 151.3 - 14	47.3	73
pDM 151.3 - 15	22.7	35
pDM 151.3 - 16	0	0
pDM 151.3 - 17	0	0
pDM 151.3 - 18	0	0
pDM 151.3 - 19	0	0
pDM 151.3 - 21	0	0
pDM 151.3 - 22	43.7	67
pDM 151.3 - 23	1.25	2
pDM 151.3 - 24	17.8	27
pDM 151.3 - 25	38	58
pDM 151.3 - 26	0	0
pDM 151.3 - 27	10.5	16
pDM 151.3 - 28	49.3	76
pDM 151.3 - 29	19.8	30
pDM 151.3 - 30	22.7	35

Example 12 - Expression of LIPOLASE®

Fifteen transformants of pDM155 are purified, cultured in shake flasks in soy/glucose medium and assayed for LIPOLASE® activity after 9 days (Table 2-see next page).

TABLE II

Transformant #	LU/ml	mg/ml
pDM 155 - 1	669	167
pDM 155 - 2	45.2	11

pDM 155 - 3	180	45
pDM 155 - 4	0	0
pDM 155 - 5	55.4	14
pDM 155 - 6	116	29
pDM 155 - 7	704	176
pDM 155 - 8	214	54
pDM 155 - 9	17.1	4
pDM 155 - 10	712	178
pDM 155 - 11	511	128
pDM 155 - 12	0	0
pDM 155 - 13	0	0
pDM 155 - 14	0	0
pDM 155 - 15	153	38
pDM 155 - 16	0	0
pDM 155 - 17	0	0
pDM 155 - 18	0	0
pDM 155 - 19	129	32
pDM 155 - 20	378	95
pDM 155 - 21	216	54

Four transformants expressed LIPOLASE® at a level of approximately 100-200 mg/l (based on the pNB assay). Transformant pDM155-10 is cultured in small scale fermentors using the conditions developed for SP387 production (see Example 9). Approximately 2.0 g/l of LIPOLASE is evident after 7 days (Figure 8A). LIPOLASE® comprised greater than 90% of secreted proteins based on SDS gel electrophoresis (Figure 8B).

DEPOSIT OF MICROORGANISMS

The following biological materials have been deposited in the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604, USA.

Strain	Accession No.	Deposit Date
E. coli containing pJRoy6	NRRL B-21285	6/20/94
E. coli containing pJRoy20	NRRL B-21418	3/10/95
E. coli containing pDM151	NRRL B-21419	3/10/95
E. coli containing pDM155	NRRL B-21420	3/10/95

The strains have been deposited under conditions that assure that access to the culture will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122 and under conditions of the Budapest Treaty. The deposit represents a biologically pure culture of each deposited strain. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.